NPC Natural Product Communications

2011
Vol. 6
No. 5
689 - 694

Antiprotozoal, Antitubercular and Cytotoxic Potential of Cyanobacterial (Blue-Green Algal) Extracts from Ireland

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Received: February 10th, 2011; Accepted: March 1st, 2011

Cyanobacteria (= blue-green algae) are prolific producers of structurally distinct and biologically active metabolites. In the continuation of our search for new sources of anti-infective natural products, we have assessed the *in vitro* antiprotozoal (*Plasmodium falciparum*, *Trypanosoma brucei rhodesiense, T. cruzi*, *Leishmania donovani*) and antitubercular (*Mycobacterium tuberculosis*) potential of samples of two terrestrial cyanobacteria, *Nostoc commune* (collected when desiccated and wet) and *Rivularia biasolettiana*. The cytotoxic potential of the extracts was also evaluated against primary L6 cells. Except for *T. cruzi* and *M. tuberculosis*, the crude extracts were active against all the organisms tested and showed no toxicity. The crude extracts were then partitioned between *n*-hexane, chloroform and aqueous methanol and retested against the same panel of pathogens. The chloroform sub-extracts of both *N. commune* samples showed significant activity against *T. b. rhodesiense* (IC_{50} values 2.0 and 3.5 μg/mL) and *P. falciparum* (IC_{50}s 7.4 and 5.8 μg/mL), with low toxicity. This trend was also true for *R. biasolettiana* extracts, and its chloroform sub-extract showed notable activity against all parasitic protozoa. There were differences in the biological activity profiles of extracts derived from desiccated and hydrated forms of *N. commune.* To our knowledge, this is the first study assessing the anti-infective activity of desiccated and hydrated forms of *N. commune*, as well as *R. biasolettiana.* Furthermore, the present work reports such biological activity in terrestrial cyanobacteria from Ireland for the first time. These results warrant the further study of Irish terrestrial cyanobacteria as a valuable source of new natural product leads for the treatment of parasitic protozoal infections.

Keywords: Cyanobacteria, *Nostoc commune, Rivularia biasolettiana, Plasmodium*, *Trypanosoma, Leishmania, Mycobacterium*.

Approximately one-third of the world's population is infected with tuberculosis (TB), the most prevalent infectious disease worldwide that kills approximately two million people annually [1]. Resistant and/or extremely drug-resistant strains of *Mycobacterium tuberculosis* are emerging in both developing and developed countries to pose a serious global health problem. Parasitic, mostly vector-borne diseases, for example, malaria, leishmaniasis and trypanosomiasis, also affect large populations in impoverished tropical regions. The most severe form of malaria is caused by the protozoan parasite *Plasmodium falciparum* and is transmitted to humans by female mosquitoes. Approximately 40% of the earth's population lives in malaria-endangered areas, and each year about 1 million people, mainly very young children, die of it [2]. The WHO estimates that more than 200 million people are at risk of either leishmaniasis or trypanosomiasis worldwide. Leishmaniasis is widespread across many tropical regions, such as India, Sudan, and Brazil. Among the three forms of leishmaniasis (cutaneous, mucosal, and visceral), the last (VL) represents the greatest threat to human health. VL is a sandfly-borne infection caused by *Leishmania donovani, L. infantum*, and *L. chagasi*. If left untreated, it can be 100% fatal within two years [3]. African trypanosomiasis (sleeping sickness), which is transmitted to humans by tsetse flies, is caused by *Trypanosoma brucei rhodesiense* and *T. b. gambiense*. It affects about half a million people on the African continent. Because the parasite is able to pass through the blood-brain barrier, the chronic phases of the disease can produce severe brain damage and death [4]. American trypanosomiasis (Chagas' disease) is endemic to 21 countries in S. America. The causative agent of the disease is *T. cruzi*, which is transmitted to humans by triatomine

insects. Chronic stage infection, if left untreated, results in neurological disorders, intestinal damage and fatal cardio-myopathy [5]. Current treatment of all these infections is limited to a few drugs, with serious drawbacks in efficacy, toxicity and cost. More importantly, drug resistance is becoming widespread and threatens their utility. These facts, combined with the absence of an efficacious vaccine and the lack of systemic vector control strategies, provide the rationale in several academic settings for the development of novel drugs against these diseases.

Cyanobacteria, an ancient photosynthetic group of prokaryotic organisms, are prolific producers of a wide range of natural products [6a-6d]. This capability has been linked with the fact that these organisms face large pressure from either grazers or competing organisms forcing them to develop chemical defense strategies for survival [7]. *Nostoc* (Nostocales, Nostocaceae) is a diverse genus of colonial cyanobacteria, commonly found in both aquatic and terrestrial habitats. The genus currently includes about 55 species [8]. *Nostoc* species, in particular *N. commune*, are well known for their capacity to withstand long periods of desiccation, cold and UV radiation that enable them to survive in most extreme environments. In dry seasons, *Nostoc* species may lie dormant for years, but readily recover when rehydrated. The dehydrated colonies, which appear as a black and brittle powder, swell into green-brown, gelatinous spheres in the presence of humidity [9]. The ability of *Nostoc* colonies to tolerate excessive environmental stress, including the long-term water deficit, has been attributed to the production of a biochemically complex, extracellular polysaccharide (glycan) matrix. The principal components of this include water-soluble (mycosporine aminoacids) and lipid-soluble (scytonemin) UV absorbing pigments, all distributed within a high molecular weight extracellular glycan unit [10].

Rivularia (Nostocales, Rivulariaceae) is a genus of about 70 species of free-living, colony-forming cyanobacteria that can be found in both marine and freshwater environments [8]. Species of the genus are cosmopolitan, often living in fast-flowing streams, seepages, salt marshes, and particularly in the intertidal of rocky shores. So far, *Rivularia* species have mainly remained unexplored chemically, and there are only few published chemical and biological activity studies.

Nostoc species have been used as both food and medicine for centuries [11]. Modern studies continue to confirm the biomedical potential of *Nostoc* species and their chemical constituents against a number of human diseases. A few previous studies compared the chemical composition (mainly fatty acids, lipids and carotenoids) of desiccated and wet *N. commune* [9,12]; however, to our knowledge, no study has been carried out previously to compare the biological activity profile of dehydrated and hydrated *N. commune* samples. The aim of this study was, therefore, to evaluate and compare the *in vitro* antiprotozoal, antitubercular and cytotoxic potential of terrestrial *N. commune* collected from Ireland when desiccated (NC-D) and when wet (NC-W). In addition, we screened another terrestrial cyanobacterium, *Rivularia biasolettiana* (RB, collected wet from Ireland) for the same biological activities. The crude extracts of the cyanobacteria were active against several protozoan parasites, and were thus partitioned between *n*-hexane, $CHCl_3$ and water, and retested against the same panel of microorganisms. Herein, we report the biological activity of the crude, as well as solvent sub-extracts obtained from three crude extracts.

The crude extracts of the cyanobacteria were tested against *P. falciparum* (erythrocytic stages of drug-resistant K1 strain), *T. brucei rhodesiense* (bloodstream forms), *T. cruzi* (intracellular amastigotes in L6 rat skeletal myoblasts), *L. donovani* (axenic amastigotes) and the tubercle bacillus (*M. tuberculosis* strain H37Rv). The toxicity of the extracts was also evaluated towards L6 cells, a primary cell line derived from mammalian (rat) skeletal myoblasts, in order to determine their selectivity. The IC_{50} and MIC values of the extracts and the reference compounds are displayed in Table 1.

None of the crude cyanobacterial extracts were active against either the tuberculosis bacillus *M. tuberculosis* or *T. cruzi* at the highest test concentrations. However, all three crude extracts had moderate to significant ability to inhibit the growth of the remaining protozoan parasites. In particular, crude extracts of both desiccated (NC-D-CR) and hydrated *N. commune* (NC-W-CR) had very promising activities against *T. brucei rhodesiense* (IC_{50} values of 3.5 and 6.6 μg/mL) and *P. falciparum* (IC_{50} values 4.3 and 4.9 μg/mL). The antileishmanial activity of both crude *Nostoc* extracts was only moderate, with the NC-D-CR extract being more active (IC_{50} 25.6 and 69.6 μg/mL). The potency of the *R. biasolettiana* crude extract (RB-CR) was also modest with IC_{50} values of 17.7 μg/mL (*P. falciparum*), 27.8 μg/mL *(T. b. rhodesiense*) and 40.4 μg/mL (*L. donovani*). Both *N. commune* extracts were found to lack any cytotoxic potential against L6 cells (IC_{50} > 90 μg/mL). The cytotoxic potential of *R. bullata* crude extract was also negligible (IC_{50} 84.3 μg/mL).

These results encouraged us to carry out a coarse separation process on the crude extracts. Hence all extracts were subjected to a liquid-liquid partition scheme to yield *n*-hexane, chloroform and aqueous methanol sub-extracts. All these semi-crude extracts were then subjected to the same *in vitro* screening panel and the results are shown in Table 1. No antimycobacterial effect was observed with any of the sub-extracts (MIC values > 256 μg/mL). None of the aqueous methanol sub-extracts had leishmanicidal activity either (IC_{50} > 100 μg/mL).

Interestingly, the most potent trypanocidal (*T. b. rhodesiense*) and plasmocidal activities were displayed by the chloroform sub-extracts of both *N. commune* samples. The same extracts also showed increased potencies, in

Table 1: Anti-protozoal, anti-mycobacterial and cytotoxic activities of Irish cyanobacteria.

Sample	*T. brucei rhodesiense*	*T. cruzi*	*L. donovani*	*P. falciparum*	*M. tuberculosis* (MIC)	Cytotoxicity L6 cells
Control drug	0.005[a]	0.41[b]	0.16[c]	0.075[d]	0.12[e]	0.009[f]
NC-D-Crude	**3.5**	>90	25.6	**4.3**	>256	>90
NC-D-Hexane	80.6	73.2	52.1	28.6	>256	>90
NC-D-$CHCl_3$	**4.0**	32.9	16.2	**5.8**	>256	39.5
NC-D-Aq.MeOH	31.8	63.8	>100	27.7	>256	>90
NC-W-Crude	**6.6**	>90	69.6	**4.9**	>256	>90
NC-W-Hexane	54.5	65.0	46.3	17.8	>256	>90
NC-W-$CHCl_3$	**2.0**	12.3	16.3	**7.4**	>256	40.5
NC-W-Aq.MeOH	74.5	71.1	>100	>50	>256	>90
RB-Crude	27.8	>90	40.4	17.7	>256	84.3
RB-Hexane	56.8	54.1	43.1	**25.4**	>256	>90
RB-$CHCl_3$	16.1	19.4	**16.4**	**14.8**	>256	47.4
RB-Aq.MeOH	>100	85.4	>100	30.7	>256	>90

All IC_{50} and MIC values are in µg/mL. IC_{50} values are mean values from at least 2 replicates of duplicates; MIC values are based on 3 replicates of triplicates (the variation is max. 20%). Control drugs: [a]melarsoprol, [b]benznidazole, [c]miltefosine, [d]chloroquine, [e]streptomycin, [f]podophyllotoxin.

comparison with the crude extracts, toward the remaining parasites *T. cruzi* and *L. donovani* (Table 1). Starting with the desiccated *N. commune* samples, NC-D-$CHCl_3$ sub-extract showed strong and comparable activities against *T. b. rhodesiense* and *P. falciparum*, with IC_{50} values of 4.0 µg/mL and 5.8 µg/mL, respectively. Unlike NC-D-CR, NC-D-$CHCl_3$ exhibited some anti-*Trypanosoma cruzi* activity (IC_{50} 32.9 µg/mL), and had almost two-fold higher antileishmanial activity (IC_{50} 16.2 µg/mL). The *n*-hexane-soluble portion of *N. commune* collected when dry (NC-D-Hexane) was active against all four protozoan species with IC_{50} values ranging between 28.6-80.6 µg/mL. As shown in Table 1, some modest trypanocidal and antimalarial activities were obtained with the aq. MeOH sub-extract (NC-D-Aq. MeOH). In general, similar trends and IC_{50} values were obtained with the wet (hydrated) *N. commune* (NC-W) sub-extracts. However, against *T. b. rhodesiense*, NC-W-$CHCl_3$ was twice as active (IC_{50} 2.0 µg/mL) than the NC-D-$CHCl_3$ sub-extract. In contrast, its antiplasmodial activity was slightly weaker (IC_{50} 7.4 µg/mL). Also noteworthy was that the NC-W-Hexane extract had slightly lower IC_{50} values against all four parasites (IC_{50} 17.8-65.0 µg/mL), whereas the NC-W-Aq. MeOH extract showed only some marginal activity against both trypanosomes with IC_{50} values around 70 µg/mL. A similar trend was also observed with *R. biasolettiana* sub-extracts. Interestingly, the RB-$CHCl_3$ sub-extract showed the best and almost identical IC_{50} values (14.8-19.4 µg/mL) against all four protozoan parasites. The *n*-hexane sub-extract (RB-Hexane) also retained generally similar IC_{50} values to those of the crude extract towards the protozoan parasites (IC_{50} values 25.4 -56.8 µg/mL), except that it had some modest potential against *T. cruzi* (IC_{50} value 54.1 µg/mL). The aq. MeOH extract (RB-Aq. MeOH) displayed some plasmocidal effect (IC_{50} 30.7 µg/mL) and very weak anti-*T. cruzi* activity (IC_{50} 85.4 µg/mL). When tested for toxicity against primary cells, only the $CHCl_3$ sub-extracts of the cyanobacterial collections exhibited weak potential with IC_{50} values around 40 µg/mL, whereas all other sub-extracts were devoid of any cytotoxicity (IC_{50} > 90 µg/mL).

In recent years, a number of cyanobacteria have been studied for their anti-infective potential to yield potent, novel natural products [6,13]. The promising biological activities and intricate structures of these metabolites have also led to several synthetic efforts [14,15]. Encouraged by these studies, we decided to screen for biological activity two terrestrial cyanobacterial species of Irish origin. One major aim of the project was to evaluate, comparatively, the biological potencies of both desiccated and hydrated forms of *N. commune*, collected from the same site at different times of the year. The results outlined here indicate that both forms seem to have similar, but still variable biological activities. This may lie in differences in their chemical compositions in different seasons of collection. Our initial TLC studies (data not shown) point out similarities in the crude and semi-crude extracts derived from both forms, but they do not appear identical. Currently, we are trying to establish more sophisticated chemical profiling techniques to compare the chemical compositions of these crude and semi-crude extracts. One trend shared by these cyanobacteria though was that the bioactivity of the crude extracts was concentrated in the $CHCl_3$ sub-extracts. This is a common experience in our laboratory, which is probably due to enrichment of secondary metabolites in this middle polarity phase after the removal of primary metabolites into the *n*-hexane (fats-lipids) and the aqueous methanol (sugars) sub-extracts.

The medicinal value of *Nostoc* species was recognized as early as 1500 BC, when they were used to treat gout, fistula and cancer [11]. Recent studies indicate their hypocholesterolemic [16], anti-inflammatory [17], antifungal [18] and acetylcholinesterase inhibitory [19] effects. Species of *Nostoc* have been a valuable source of biologically-active compounds, such as the cryptophycins with potent anticancer activity. These potent tubulin inhibitors have served as template compounds for a semi-synthetic clinical candidate [20]. A number of other bioactive peptides, diterpenes and alkaloids have been reported from *Nostoc* species with cytotoxic, antimicrobial, antiviral and antiprotozoal activities [6,20]. Nostocarboline, a quaternary beta-carbolinium alkaloid derived from a *Nostoc* sp. and its synthetic derivatives

show potent antiprotozoal and antimycobacterial activities [14,15]. The hydrated forms of *Nostoc* species, particularly *N. commune* are consumed as foodstuff, primarily in Asia and the Andes [21a,b]. It is a rich source of protein, fat, carbohydrate, crude fiber and vitamins [22a,b]. However a recent study showed the presence of beta-methylamino-L-alanine (BMAA), a neurotoxic aminoacid in *Nostoc* species [21a]. It would be of interest to test whether our Irish *N. commune* samples contain BMAA.

A literature survey indicates that *Rivularias* species have been poorly investigated. Several bromoindole alkaloids have been isolated from *R. firma* [23a,b], and some *Rivularia* species have been studied for their fatty acid [24a,b] and sugar constituents [25]. Antibacterial, antifungal [24b,26] and algicidal [27] effects of some species have also been assessed. To our knowledge, this is the first study evaluating antiprotozoal, antitubercular and cytotoxic potential of a *Rivularia* species.

The present investigation indicated the *in vitro* antiprotozoal potential of terrestrial cyanobacteria collected in the wild from Ireland. In particular, the chloroform-soluble portion of these extracts displayed highly interesting antiprotozoal activity. Because of the promising IC_{50} values and low toxicity, these extracts of cyanobacteria, particularly the chloroform sub-extracts, merit further investigation for the isolation and characterization of their active metabolites. This is the subject of our current research. Antimalarial and antiprotozoal activities of some alkaloids obtained from *Nostoc* species have been reported [14,15], but this is the first study comparing the antiprotozoal activity of *N. commune* collected in desiccated and hydrated forms. It also appears that the current work represents the first antiprotozoal, antitubercular and cytotoxic assessment of any *Rivularia* species. To our knowledge, this is also the first study assessing such biological activity of Irish cyanobacteria.

Experimental

Cyanobacterial samples: All cyanobacterial samples were collected in the Burren near Finavarra, Co. Clare, Ireland. The dry form of *Nostoc commune* Vaucher ex Bornet & Flahault was collected in May 2007, whereas *Rivularia biasolettiana* Meneghini ex Bornet and the wet form of *N. commune* were collected in November and December 2007, respectively. Identifications were made by one of us (M.G.) based on Whitton [28]. Voucher samples of *N. commune* are lodged in the Herbarium of the Hampshire County Council Museums Service in Winchester, Hampshire (accession number Bi 2000. 16. 375), and of both *N. commune* and *R. biasolettiana* at the University of London, School of Pharmacy (voucher # GB07-C1 through GB07-C3).

Extraction and partition: The collected cyanobacteria were immediately placed in *i*-PrOH and stored at -20˚C in a freezer until work-up. Algal material was homogenized in a household blender and filtered. The residue was extracted overnight with $CHCl_3$: MeOH mixtures (3:1 and 1:1) under continuous stirring at room temperature and combined with the initial *i*-PrOH extract obtained above. The combined crude extract was dissolved in 10 mL EtOAc: MeOH (1:1) and centrifuged to remove insoluble materials. The supernatant was removed, evaporated to dryness *in vacuo* at 30˚C, and used for the initial biological assays. A portion of each crude extract (1 g) was dissolved in 10% water in MeOH (100 mL) and partitioned against *n*-hexane (3x100 mL). The water content of the MeOH phase was then adjusted to 30% by adding water before partitioning against $CHCl_3$ (3x100 mL). The *n*-hexane and $CHCl_3$ extracts were evaporated to dryness *in vacuo* at 30˚C, whereas the aq. MeOH sub-extract was freeze-dried before using in the biological assays.

Trypanocidal activity against T. brucei rhodesiense and cytotoxicity: Minimum Essential Medium (50 µL) supplemented with 25 mM HEPES, 1g/L additional glucose, 1% MEM non-essential amino acids (100 x), 0.2 mM 2-mercaptoethanol, 1mM Na-pyruvate and 15% heat inactivated horse serum was added to each well of a 96-well microtiter plate [29]. Serial drug dilutions of seven 3-fold dilution steps (90 - 0.123 µg/mL) were prepared. Then 10^4 bloodstream forms of *T. b. rhodesiense* STIB 900 in 50 µL was added to each well and the plate incubated at 37°C under a 5% CO_2 atmosphere for 72 h. Ten µL Alamar Blue was then added to each well and incubation continued for a further 2-4 h. The plates were read with a Spectramax Gemini XS microplate fluorometer using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Data were analyzed using the microplate reader software Softmax Pro (Molecular Devices Cooperation, Sunnyvale, CA, USA). Cytotoxicity was assessed using the same assay on rat skeletal myoblasts (L6 cells). Melarsoprol and podophyllotoxin were the control drugs.

Trypanocidal activity against T. cruzi: Rat skeletal myoblasts (L6 cells) were seeded in 96-well microtiter plates at 2000 cells/well in 100 µL RPMI 1640 medium with 10% FBS and 2 mM l-glutamine. After 24 h, the medium was removed and replaced by 100 µL per well containing 5000 trypomastigote forms of *T. cruzi* Tulahuen strain C2C4 containing the β-galactosidase (Lac Z) gene [30]. After 48 h, the medium was removed from the wells and replaced by 100 µL fresh medium with or without a serial drug dilution of seven 3-fold dilution steps. After 96 h of incubation the plates were inspected under an inverted microscope to assure growth of the controls and sterility. The substrate CPRG/Nonidet (50 µL) was added to all wells. A color reaction developed within 2-6 h, which was read photometrically at 540 nm. Data were transferred into the graphic program Softmax Pro, which calculated IC_{50} values. Benznidazole was the standard drug used.

Leishmanicidal activity against L. donovani: Amastigotes of *L. donovani* strain MHOM/ET/67/L82 were grown in

axenic culture at 37°C in SM medium at pH 5.4 supplemented with 10% heat-inactivated fetal bovine serum under an atmosphere of 5% CO_2 in air. Culture medium (100 mL) with 10^5 amastigotes from axenic culture with or without a serial drug dilution were seeded in 96-well microtiter plates. Serial drug dilutions were prepared, and after 72 h of incubation the plates were inspected under an inverted microscope to assure growth of the controls and sterile conditions. Ten µL of Alamar Blue was then added to each well and the plates incubated for another 2 h [31]. Then the plates were read with a Spectramax Gemini XS microplate fluorometer using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Data were analyzed using the software Softmax Pro. Decrease of fluorescence (= inhibition) was expressed as percentage of the fluorescence of control cultures and plotted against the drug concentrations. From the sigmoidal inhibition curves the IC_{50} values were calculated. Miltefosine was used as a reference drug.

Antimalarial activity against P. falciparum: *In vitro* activity against erythrocytic stages of *P. falciparum* was determined by a modified [^{3}H]-hypoxanthine incorporation assay using drug-resistant K1 strain and the standard drug chloroquine. Briefly, parasite cultures incubated in RPMI 1640 medium with 5% Albumax (without hypoxanthine) were exposed to serial drug dilutions in microtiter plates. After 48 h of incubation at 37°C in a reduced oxygen atmosphere, 0.5 µCi ^{3}H-hypoxanthine was added to each well. Cultures were incubated for a further 24 h before they were harvested onto glass-fiber filters and washed with distilled water. Radioactivity was measured using a Betaplate™ liquid scintillation counter (Wallac, Zurich). The results were recorded as counts per min (CPM) per well at each drug concentration and expressed as a percentage of the untreated controls. IC_{50} values were calculated from graphically plotted dose-response curves.

Antitubercular activity against M. tuberculosis: Minimum inhibitory concentrations (MICs) were measured using an MTT assay based on the method detailed by Montoro *et al.* [32]. Briefly, Middlebrook 7H9 medium (100 µL), supplemented with 10% oleic acid-albumin-dextrose-catalase supplement, 0.5% glycerol and 0.005% tween 80 was added to each well of a 96-well flat-bottom plate. Serial drug dilutions were added to the wells (final concentrations of 1-256 µg/mL) and approximately 2.5 x 107 *M. tuberculosis* strain H37Rv in 100 µL was added to each well. The plate lid was then sealed with parafilm and the plate incubated with gentle rocking (30 oscillations per min) for 7 days at 37°C. Ten µL MTT (filter-sterilized at 5 mg/mL in dH_2O) was added to each well and the plates were then incubated for a further 24 h. MICs were recorded as the lowest concentration at which a purple precipitate of formazin did not appear in the wells. Streptomycin was used as a positive control.

Acknowledgments – This project was partly supported by the University of London Central Research Funds (DT), together with funding from the Beaufort Marine Biodiscovery Programme co-ordinated by the Marine Institute (Ireland). We thank Alan and Anne Greenhouse for help with the collection of the May sample of *N. commune.*

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